

Research Note—

Enhanced Innate Immune Responses in a Brood Parasitic Cowbird Species: Degranulation and Oxidative Burst

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SUMMARY. We examined the relative effectiveness of two innate immune responses in two species of New World blackbirds (Passeriformes, Icteridae) that differ in resistance to West Nile virus (WNV). We measured degranulation and oxidative burst, two fundamental components of phagocytosis, and we predicted that the functional effectiveness of these innate immune responses would correspond to the species' relative resistance to WNV. The brown-headed cowbird (*Molothrus ater*), an obligate brood parasite, had previously shown greater resistance to infection with WNV, lower viremia and faster recovery when infected, and lower subsequent antibody titers than the red-winged blackbird (*Agelaius phoeniceus*), a close relative that is not a brood parasite. We found that cowbird leukocytes were significantly more functionally efficient than those of the blackbird leukocytes and 50% more effective at killing the challenge bacteria. These results suggest that further examination of innate immunity in the cowbird may provide insight into adaptations that underlie its greater resistance to WNV. These results support an eco-immunological interpretation that species like the cowbird, which inhabit ecological niches with heightened exposure to parasites, experience evolutionary selection for more effective immune responses.

RESUMEN. *Nota de Investigación*—Aumento de la respuesta inmune inata en tordos cabecicafés, parásitos de la puesta: Degranulación y estallido oxidativo.

Se examinaron la efectividad relativa de dos respuestas inmunes inatas en dos especies de tordos del Nuevo Mundo (Passeriformes, Icteridae), que difieren en la resistencia contra el virus del Nilo Oriental. Se midieron la degranulación y el estallido oxidativo, dos componentes fundamentales de la fagocitosis y se predijo que la efectividad funcional de estas respuestas inmunes inatas corresponde a la resistencia relativa al virus del Nilo Oriental. El tordo cabecicafé (*Molothrus ater*), un parásito de la puesta obligado, había mostrado una resistencia mayor a la infección por este virus, con una viremia menor y una recuperación más rápida de la infección y la presencia de títulos más bajos en comparación con el tordo sargento (*Agelaius phoeniceus*), un pariente cercano que no es un parásito de la puesta. Se encontró que los leucocitos fueron más eficientes funcionalmente de manera significativa en comparación con los observados en los tordos sargentos y eran 50% más efectivos para neutralizar las bacterias de desafío. Estos resultados sugieren que un posterior análisis de la inmunidad inata en el tordo cabecicafé puede proveer información sobre las adaptaciones en que se basa su resistencia mayor al virus del Nilo Oriental. Estos resultados apoyan una interpretación ecológica e inmunológica sobre especies como el tordo cabecicafé, que habita en nichos ecológicos con una exposición aumentada a parásitos y que experimenta una selección evolutiva para montar respuestas inmunes más efectivas.

Key words: innate immunity, oxidative burst, degranulation, eco-immunology, cowbird, brood parasite, West Nile virus

Abbreviations: CFU = colony-forming units; DCFHDA = dichlorofluorescein diacetate; EDTA = disodium ethylenediamine-tetraacetic acid; OpSE = opsonized *Salmonella* Enteritidis; PBS = phosphate-buffered saline; PMA = phorbol myristate; RFU = relative fluorescence units; RMPI = Roswell Park Memorial Institute; WEEV = western equine encephalomyelitis virus; WNV = West Nile virus

The emergence of West Nile virus (*Flaviviridae*, *Flavivirus*, WNV) in North America in 1999 revealed marked differences in illness and mortality among avian species, including high susceptibility of songbirds (Passeriformes) to infection and death (20,25,33). Interestingly, among songbirds, the New World cowbirds (genus *Molothrus*), all obligate brood parasites, are unusually resistant to WNV infection (17,32). Cowbirds showed lower viremia when infected by WNV and faster recovery than related species that are not brood parasites (10). Cowbirds' refractoriness to infection with WNV resembles species in taxonomically distant orders such as doves (Columbiformes), parrots (Psittaciformes), and chickens (Galliformes) more than other songbird species (Passeriformes) (30,31,32). Since avian variation in susceptibility to WNV occurs

primarily at or above the taxonomic level of family (17), the cowbird anomaly invited examination of its immune responses.

We compared immune responses of the brown-headed cowbird (henceforth "cowbird") (24) with those of the red-winged blackbird (henceforth "blackbird") (44), a related species that is not a brood parasite and is more susceptible to WNV infection. The cowbird and blackbird are closely related species in the family Icteridae, the New World blackbirds (16), and they are in the same branch of the family, grackles and allies (22), as well as being similar in many aspects of behavior and ecology (7).

We examined two innate immune defenses that are fundamental components of phagocytosis (37), oxidative burst and degranulation. These are specific functional killing mechanisms that can be measured by assays that are highly reliable (42). In oxidative burst, killing occurs by an oxygen-dependent process that uses reduced nicotinamide adenine dinucleotide phosphate and produces reactive

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oxygen species to destroy the engulfed material (18,19). In degranulation, killing occurs by an oxygen-independent process that depends on the release of granules to destroy or deprive the engulfed particles of material needed for growth (14,37). Innate immune responses are known to be involved in the recognition of WNV and activation of adaptive immune mechanisms (2,6), and innate immunity is likely to play an important role in the cowbird's resistance to WNV specifically, since viremia is nondetectable in less than a week (20,32) and such rapid control is characteristic of innate immunity (15).

We predicted that the cowbird would show significantly greater functional effectiveness of both oxidative burst and degranulation responses than the blackbird, a difference that would correspond to the cowbird's greater resistance to WNV.

MATERIALS AND METHODS

Species. Brown-headed cowbird is a small seed-eating songbird, 17.5 cm long and 40–50 g (24,27,38). An obligate brood parasite, the cowbird does not build nests, but always lays its eggs in the nests of other avian species. These foster parent species incubate the cowbird eggs and raise the young (4,34). We worked with *M. ater obscurus*, a subspecies whose range is in the southwestern United States and along the Pacific Coast (24).

Red-winged blackbird is also a small seed-eating species, slightly larger than the cowbird (20.5 cm, 55–65 g) (38), similar in ecology, behavior, and breeding system except for the habit of brood parasitism (7). Red-winged blackbirds nest in wet, brushy, and marshy areas as well as adjacent upland fields, and they feed in these habitats as well as in grasslands and agricultural areas (44).

Study site and fieldwork. The study site lies on the Edwards Plateau in Texas, a mixed ash-juniper and oak woodlands habitat with interspersed grasslands (21). The study was done in January 2009, in Fort Hood, Texas, in conjunction with a cowbird control project that protects two endangered species, golden-cheeked warbler (*Dendroica chrysoparia*) and black-capped vireo (*Vireo atricapilla*) from cowbird nest parasitism (11). We collected 0.15 ml of blood from each of 30 brown-headed cowbirds and 30 red-winged blackbirds after they were trapped in large walk-in traps baited with white millet. Both species were trapped and handled under appropriate state and federal permits: Title 50, Code of Federal Regulations, part 21.43 (Depredation order for blackbirds, cowbirds, grackles, crows and magpies; Title 50 CRF 21.12, use by public institutions for research purposes). Protocols were approved by Patuxent Wildlife Research Center Animal Care and Use Committee and United States Department of Agriculture-Agricultural Research Service (USDA-ARS)-Southern Plains Agricultural Research Center.

Immune response assays. Whole blood assays that measure functional effectiveness of leukocytes were carried out within 2–3 hr of blood collection at the Southern Plains Agricultural Research Center, College Station, TX. Methodology for assays to measure oxidative burst and degranulation is described by Koguy *et al.* (18,19). The oxidative burst assay uses chemiluminescence to measure the release of toxic oxygen radicals made by the phagocytic leukocytes. The degranulation assay measures the release of a primary enzyme (β -D-glucuronidase) that is stored in preformed granules in the host cytoplasm (1).

Blood was collected from each bird and placed in plastic vacutainer tubes spray coated with disodium ethylenediaminetetraacetic acid (K_2 EDTA; BD Vacutainer, Franklin Lakes, NJ), then tubes were shaken vigorously to prevent clotting and to mix thoroughly. For both species, the blood from three separate groups of birds was combined, and independent assays were carried out. Each pool was transferred to a conical tube, and 30 ml of 1% methylcellulose prepared in Roswell Park Memorial Institute (RPMI) 1640 medium was added, mixed thoroughly, and centrifuged at $35 \times g$ for 20 min at 4 C. The supernatant containing the peripheral blood leukocytes, including heterophils and monocytes, was transferred to a new conical tube and 10 ml of clear

RPMI medium was added; then cells were pelleted by centrifugation ($485 \times g$ for 15 min at 4 C). The cells were resuspended (1×10^9 /ml) in fresh RPMI medium (1 ml).

Oxidative burst assay. Production of an oxidative burst by phorbol myristate (PMA)-stimulated peripheral blood leukocytes was measured for the two species by oxidation of nonfluorescent 2',7'-dichlorofluorescein diacetate (DCFHDA) to fluorescent 2',7'-dichlorofluorescein as described previously (12) with modification. One milliliter of leukocytes (8×10^6 cells/ml) was added to 2-ml microcentrifuge tubes, then incubated with PMA (1.62 μ M) and DCFHDA (10 μ g/ml in final concentration) for 1 hr at 37 C. The aliquots of cell cultures (150 μ l) were then dispensed to a black 96-well plate, and the fluorescence was measured using a GENios Plus Fluorescence Microplate Reader (TECAN US Inc., Research Triangle Park, NC) at 485-nm excitation and 530-nm emission wavelengths. The relative fluorescent units (RFUs) were recorded after 60 min.

Degranulation assay. A poultry isolate of *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) (97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA). *Salmonella* Enteritidis is a highly pathogenic bacterium that serves as a useful challenge pathogen in stimulating immune responses.

Salmonella Enteritidis was cultured in tryptic soy broth (Difco Laboratories, Becton Dickinson Co., Sparks, MD) overnight at 41 C. The bacteria were pelleted ($7700 \times g$, 10 min), washed with cold phosphate-buffered saline (PBS) and centrifuged again ($7700 \times g$, 10 min). The supernatant was discarded, and the pellet was resuspended in 1 ml of cold PBS. A stock solution of *Salmonella* Enteritidis (1×10^9 colony-forming units [CFU]/ml) was prepared in PBS. The bacterial concentration was determined spectrophotometrically (Spectronic 20D spectrophotometer; Milton Roy Co., Golden, CO) using a standard curve with a reference wavelength of 625 nm. *Salmonella* Enteritidis was prepared fresh for each experiment and kept on ice until used.

Opsonized *Salmonella* Enteritidis (OpSE) was prepared as previously described (8). Briefly the bacteria (1×10^9 CFU/ml) were suspended in normal chicken serum (4:1 [v:v]) and incubated for 60 min at 39 C on a rotary shaker. OpSE was stored at 4 C until used in the degranulation assays.

Degranulation was measured by quantifying β -D-glucuronidase activity (19) in culture medium (RPMI without serum; 1 ml [8×10^6 /ml]) following stimulation of leukocytes (8×10^6 /ml) with OpSE at 37 C for 1 hr on a rocker platform in 5% CO₂ and 95% humidity. After incubation, the cells were pelleted by centrifugation at $10,000 \times g$ for 2 min at 4 C, and supernatants were collected for the assay. An aliquot of 25 ml of supernatant was incubated with 50 ml of freshly prepared substrate (10 mM 4-methylumbelliferyl β -D-glucuronide and 0.1% Triton X-100 in 0.1 M sodium acetate buffer) in a black 96-well plate for 4 hr at 37 C. The reaction was stopped by adding 200 μ l of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4) to each well. Liberated 4-methylumbelliferone was measured fluorometrically (355/460 nm) using a fluorescence microplate reader (Genios Plus Plate Reader, TECAN US Inc.).

Statistical analysis. The data from the repeated experiments were combined for presentation and statistical analysis. Statistical differences between treatment groups were determined by ANOVA ($P < 0.05$). Means were further separated for significance with a pair-wise multiple comparison procedure (Tukey test, $P < 0.05$). Significant differences were further separated using Duncan's multiple range test.

RESULTS

The oxidative burst assay, which measures toxic oxygen radicals available to kill bacteria, and the degranulation assay, which measures a primary enzyme available in host cytoplasmic granules, showed that cowbird phagocytic leukocytes were significantly more functionally efficient than those of the red-winged blackbird leukocytes and >50% more effective at killing the challenge bacteria, *Salmonella* Enteritidis.

Table 1. Measurement of innate immune response: oxidative burst assay.

Species ^A	No. of birds	Treatment	Mean relative RFU ^{BC}
Controls			
1. Red-winged blackbird	30	Control (medium only)	7.46 ± 0.15 ^a
2. Brown-headed cowbird	30	Control (medium only)	7.88 ± 0.12 ^a
Experimental challenge			
1. Red-winged blackbird	30	PMA	19.83 ± 0.27 ^b
2. Brown-headed cowbird	30	PMA	42.7 ± 0.48 ^c

^ACell source is total peripheral blood leukocytes.^BData presented as $\times 10^3 \pm$ standard error of the mean.^CDifferent superscripts indicate significant differences at $P \leq 0.001$.

Leukocyte functional assays. *Oxidative burst assay.* Leukocytes from brown-headed cowbirds, stimulated with PMA, produced a concentration-dependent oxidative burst that was significantly greater than that of leukocytes stimulated with PMA from red-winged blackbirds: 42.7 ± 0.48 RFU *vs.* 19.83 ± 0.27 RFU; $P < 0.001$) (Table 1). No significant differences were found between nonstimulated (control) leukocytes isolated from cowbirds and red-winged blackbirds.

Degranulation assay. Leukocytes from cowbirds, stimulated with OpSE, induced a concentration-dependent release of the primary granule, β -D-glucuronidase, that was significantly greater than that from leukocytes of red-winged blackbirds: 15.752 ± 0.603 μ M *vs.* 9.945 ± 0.641 μ M ($P < 0.001$; Table 2). There was also a difference between nonstimulated (control) leukocytes isolated from cowbirds and red-winged blackbirds (9.621 ± 0.491 μ M *vs.* 6.698 ± 0.736 μ M; Table 2): the nonstimulated leukocytes of cowbirds showed greater release of the primary granule β -D-glucuronidase than did the nonstimulated leukocytes of blackbirds.

DISCUSSION

The scale of difference of the oxidative burst and degranulation responses reported here for the cowbird and the blackbird is noteworthy and comparable to the functional immune differences observed between two distinct genetic lines of chickens (41,42). These results suggest that further examination of innate immune responses may be a fruitful area for understanding the basis for the cowbird's greater resistance to WNV.

This study also identified a subtle distinction between the relative effectiveness of the two immune responses. The cowbird's degranulation response was higher under both control and experimental conditions (Table 2), while its oxidative burst response was higher only under experimental conditions (Table 1). These two systems typically operate simultaneously in the organism, but our results highlight that these immune defenses can evolve independently, perhaps depending on the mix of pathogenic challenges found in a particular ecological niche. In phagocyte biology,

oxidative burst response is considered to address oxygen-dependent microbial challenges, while the degranulation addresses oxygen-independent microbial challenges.

Finally, the results reported here also are relevant to whether cowbirds utilize innate immune response more than adaptive immune responses compared with species that are not brood parasites. Reisen and Hahn (32) reported that the cowbird exhibited significantly lower antibody titers for WNV postinfection than three species of blackbirds that are not brood parasites. Our results in this study encourage further examination of cowbird immunity to investigate whether the cowbird lowers the energetic costs of its immune responses by lower activation of adaptive immune responses.

The unusual life history strategy of brood parasitism exhibited by the cowbird makes it well-suited to test the hypothesis that parasite-rich environments favor evolution of more effective immune defenses (13,23,29), a topic of interest in the emerging area of eco-immunology (26,35,36). Brood parasites, in effect, inhabit a pathogen-rich ecological niche, because they are exposed to and infected by the diverse parasites associated with their foster parent species—like species-specific lice (9). The brown-headed cowbird's more effective resistance to WNV is not a single-species phenomenon, but also characterizes shiny cowbird (*M. bonariensis*) and bronzed cowbird (*M. aeneus*) (10), and its more effective immunity extends to other arboviruses, including western equine encephalitis (*Togaviridae*, *Alphavirus*, WEEV), St. Louis encephalitis (*Flavivirus*, *Flaviviridae*), and a challenging co-infection with WNV and WEEV (32).

The two assays used in this study represent a technological advance in immune studies of wild species. As far as we are aware, no other studies of wild birds have been done using the degranulation assay. Use of *Salmonella* Enteritidis, a highly pathogenic bacterium, permits assessment of immune responses to a real-world pathogen, unlike the nonpathogenic bacterial challenges frequently used in eco-immunological studies (26). The oxidative burst assay has previously been used to demonstrate differences in immune responses in different treatment groups and different species of wild birds

Table 2. Measurement of innate immune response: degranulation assay.

Species ^A	No. of birds	Treatment	β -D-glucuronidase released (μ M) ^{BC}
Controls			
1. Red-winged blackbird	30	Control (medium only)	6.698 ± 0.736 ^a
2. Brown-headed cowbird	30	Control (medium only)	9.621 ± 0.491 ^b
Experimental challenge			
1. Red-winged blackbird	30	OpSE	9.945 ± 0.641 ^b
2. Brown-headed cowbird	30	OpSE	15.752 ± 0.603 ^c

^ACell source is total peripheral blood leukocytes.^BData presented as mean μ M \pm standard error of the mean.^CDifferent superscripts indicate significant differences at $P \leq 0.001$.

(28,39), but the present study is the first to demonstrate use of PMA. Both the degranulation and oxidative burst assays are more specific than the bacterial killing assay, one of the most widely used assays in eco-immunological studies (5,40). They also permit reliable comparisons among species (3,43) as well as comparisons with general killing assays such as the bacterial killing assay.

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